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Role of 6-methoxybenzoxazolinone (6MBOA) in maize inbred resistance to first brood European corn borer

Jerome Anthony Klun
Iowa State University

ROLE OF 6-METHOXYBENZOXAZOLINONE (6MBOA) IN MAIZE INBRED RESISTANCE TO FIRST BROOD EUROPEAN CORN BORER

by

Jerome Anthony Klun

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Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

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INTRODUCTION

The resistance of cultivated plants to insects greatly influences the yield and quality of crops. Among the same plant species, varieties or strains are known to differ widely in degree of resistance exhibited. Plant breeders have been able to develop new varieties more resistant than before and have made rapid progress in expanding the knowledge of inheritance and genetics of host plant resistance. In particular, advancements by corn breeders have been highly significant as is evidenced by the development of strains resistant to the European corn borer, Ostrinia nubilalis (Hübner). However, the chemical nature of host plant resistance is rather obscure.

Early workers have associated maize inbred resistance to the first brood European corn borer with the compound 6-methoxybenzoxazolinone. In addition, various corn extract fractions have been demonstrated to contain borer growth inhibitors or toxins but, these have not yet been identified chemically. Preliminary studies up to this time have been, of necessity, qualitative in nature.

Workers in the field of host plant resistance have long indicated that quantitative identification of a plant chemical or chemicals directly or indirectly associated with the resistance of maize to the European corn borer

would enhance the knowledge of host plant resistance on a biochemical basis and provide a chemical marker for resistance which would facilitate genetic study of the phenomenon. To this end, the following research was conducted.

REVIEW OF LITERATURE

The history of maize resistance to the European corn borer has been reviewed by George (1957). Dicke (1954), Penny and Dicke (1957), and Guthrie et al. (1960) have elaborated on the genetics of resistance and methods of rating maize plants into classes according to degree of resistance exhibited to the borer.

Considerable work has been carried out so as to characterize and identify plant chemicals which exhibit insecticidal and antimicrobial properties. Artturi I. Virtanen of Helsinki, Finland and his co-workers have isolated, characterized, and synthesized a number of antifungal substances from extracts of rye (Virtanen and Hietala 1955), potato (Valle 1957), red clover (Virtanen and Hietala 1958), wheat and maize (Virtanen et al. 1956). That compound isolated from wheat and maize was 6-methoxybenzoxazolinone. The substance 6-methoxybenzoxazolinone (6MBOA) was first found in extracts of plant tissues of Coix by Koyama et al. (1955). About the same time it was isolated from maize extract by Loomis (1956). The work of Loomis was carried out independently and nearly simultaneously with that of Virtanen et al. (1956) and came about as an outgrowth of earlier findings reported by Beck (1951). Beck indicated that young corn plants contain substances which inhibit the growth of the European corn

borer larvae. Subsequent work by Hietala and Wahlroos (1956) and Smissman et al. (1957a) (1957b) confirmed the earlier characterization of this compound. Detailed investigations by Beck and Stauffer (1957) disclosed the presence of three borer growth inhibitors in corn tissue. One of these inhibitors was 6MBOA. The second and third inhibitors, although isolated, were not characterized. These growth inhibitors were termed Resistance Factor A, B, and C respectively. The compound 6MBOA has been demonstrated to be a growth inhibitor not only of the European corn borer but also of bacteria, fungi, and other insects. (Beck and Stauffer 1957a), and (Wahlroos and Virtanen 1958).

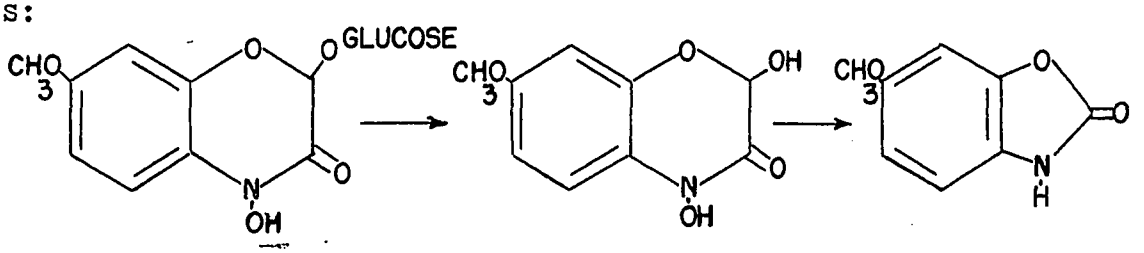
Bioassay tests with the European corn borer and Penicillium were utilized by Beck (1957) to determine resistance factor activity in whorl leaf tissue of five dent corn inbreds; WF9, W204, W210D, W22, and W22R. He found there was significantly more resistance factor activity only in W210D as compared to the standard susceptible line WF9. Beck et al. (1957b) reported a method for estimating the concentration of 6MBOA in corn plant tissue extracts. In the analysis, plant tissue is extracted with diethyl ether, and the ether extract is purified by column chromatography with aluminum oxide. The compound is eluted from the alumina column by an ether-alchol solvent mixture, and its concentration is estimated by its extinction at 285 mu.

The technique afforded a 91 per cent average recovery of 6MBOA as determined from a series of recovery experiments utilizing pure 6MBOA placed on similar columns. It was reported the concentration for fresh tissues of plant parts, varieties, ages, vary from 3 to 260μg/gram fresh tissue.

Beck (1961) tested chemical analogs of 6MBOA for biological activity upon the European corn borer and Penicillium chrosogenum. "Fungal growth inhibition was found to be dependent on two characteristics of the molecule (1) the presence of lipid-solubilizing group on the benzenoid nucleus and (2) the presence of nitro or amino group adjacent to a phenolic hydroxyl. Antifungal activity was not enhanced by the oxazole ring." However, inhibition of the European corn borer was closely associated with the presence of an oxazole or thiazole grouping while phenolic compounds were of low activity.

In 1959 it was reported by Wahlroos and Virtanen (1959) that 6MBOA was not an in vivo consituent of corn tissue. The same workers demonstrated a glucoside, 4-O-glucosyl-2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, to be an in vivo precursor of 6MBOA both in wheat and maize (Wahlroos and Virtanen 1959). According to Virtanen's group, when the corn or wheat plant is crushed, the glucoside is rapidly hydroyzed by glucosidase to glucose and the corresponding aglucone, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one. The aglucone

is quantitatively converted to 6MBOA by heating in an aqueous solution. (Wahlroos and Virtanen 1959). The mechanism of the aglucone degradation was elaborated upon by Brendenberg et al. (1962). Schematically the reaction is:



On the basis of the evidence before him, Virtanen (1961) felt it improbable that 6MBOA could be the in vivo toxic factor which causes high mortality among European corn borer larvae on certain inbred corn tissues. "Since the aglucone is rapidly formed from the glucoside precursor in the presence of glucosidase, injury of the corn tissue by the borer would effect this reaction and thereby borer larvae would consume the aglucone". He concluded, "It is quite possible that this aglucone may the Resistance Factor A. The possibility still exists that the aglucone is converted into 6MBOA in the intestinal canal of the larvae, but as this has not been experimentally demonstrated, the aglucone is most probably the active factor".

The in vivo absence of 6MBOA was not accepted by Smissman et al. (1963) and the group demonstrated 6MBOA to occur in vivo. Virtanen and Wahlroos (1963) criticized the techniques utilized by Smissman et al. (1963) to demonstrate

the *in vivo* occurrence of 6MBOA and reaffirmed their position that 6MBOA is not found *in vivo*.

Reimann and Byerrum (1964) have studied the biosynthesis of the aglucone. The purpose of their study was to obtain information concerning the mechanism of benzoxazine and cyclic hydroxamate formation in higher plants. It was concluded that the aromatic ring of the aglucone is biosynthesized from an intermediate derived from shikimic acid, and the O-methyl group is formed by a transmethylation reaction from methionine and the heterocyclic ring carbons are derived from an intermediate formed from the 1 and 2 carbons of ribose.

Roth and Knusli (1961) and Hamilton and Moreland (1962) have demonstrated the aglucone and glucoside precursors of 6MBOA to be the active agents of corn sap which effects the *in vitro* detoxification of a triazine herbicide, 2-chloro-4,6-bis-ethylamino-S-triazine. Discovery of this phenomenon was nurtured by the effort of investigators to explain the resistance of corn plants to the herbicide. Hamilton (1964) has carried out a genetic study of the tolerance of corn to the triazine herbicide. He concluded that a decrease in tolerance to the herbicide is associated with low benzoxazinone content and that the character (low benzoxazinone content) behaves as a single recessive gene upon inbreeding.

As mentioned earlier, 6MBOA and its glucoside and

aglucone precursors are also found in wheat. It is of interest to note that Elnaghy and Linko (1962) have associated the glucoside with the resistance of wheat to fungus.

MATERIALS AND METHODS

The primary objective of this research was to evaluate the role of 6-methoxybenzoxazolinone in the resistance of maize inbreds to the first brood European corn borer, first by determining if there was a functional correlation between the amount of 6-methoxybenzoxazolinone produced by a maize inbred series and the regression of host plant resistance observed in that inbred series, and secondly by determining the biological activity of the compound through bioassay tests.

Isotope Dilution Technique

A prerequisite to the research was the development of a quantitative method of analysis for the compound in question. It is evident that the quantitative isolation of a single pure constituent from an extremely complex organic and inorganic system is highly improbable since in most analyses, purity and quantitative isolation are mutually contradictory requirements. Where simple quantitative chemical separations are not available it is often feasible to exploit the intrinsic property of radioisotopes by the application of the isotope dilution technique. The principle of this method may be briefly defined as follows: In order to analyze a sample of a given size

containing X grams of a specific compound, Y grams of the isotopic form of the compound with a specific activity SA_1, is added to the sample to be assayed. After the isotopic form of the compound has been completely distributed through the system being analyzed, a small amount of pure compound is isolated and its final specific activity SA_2 determined. Since the total activity for the system remains constant, the following relationships hold:

Weight	Specific Activity
X grams of compound	0
Y grams of labeled compound	SA_1
(X + Y) grams of mixture	SA_2

Thus,

$$\frac{Y}{(X+Y)} = \frac{SA_2}{SA_1}$$

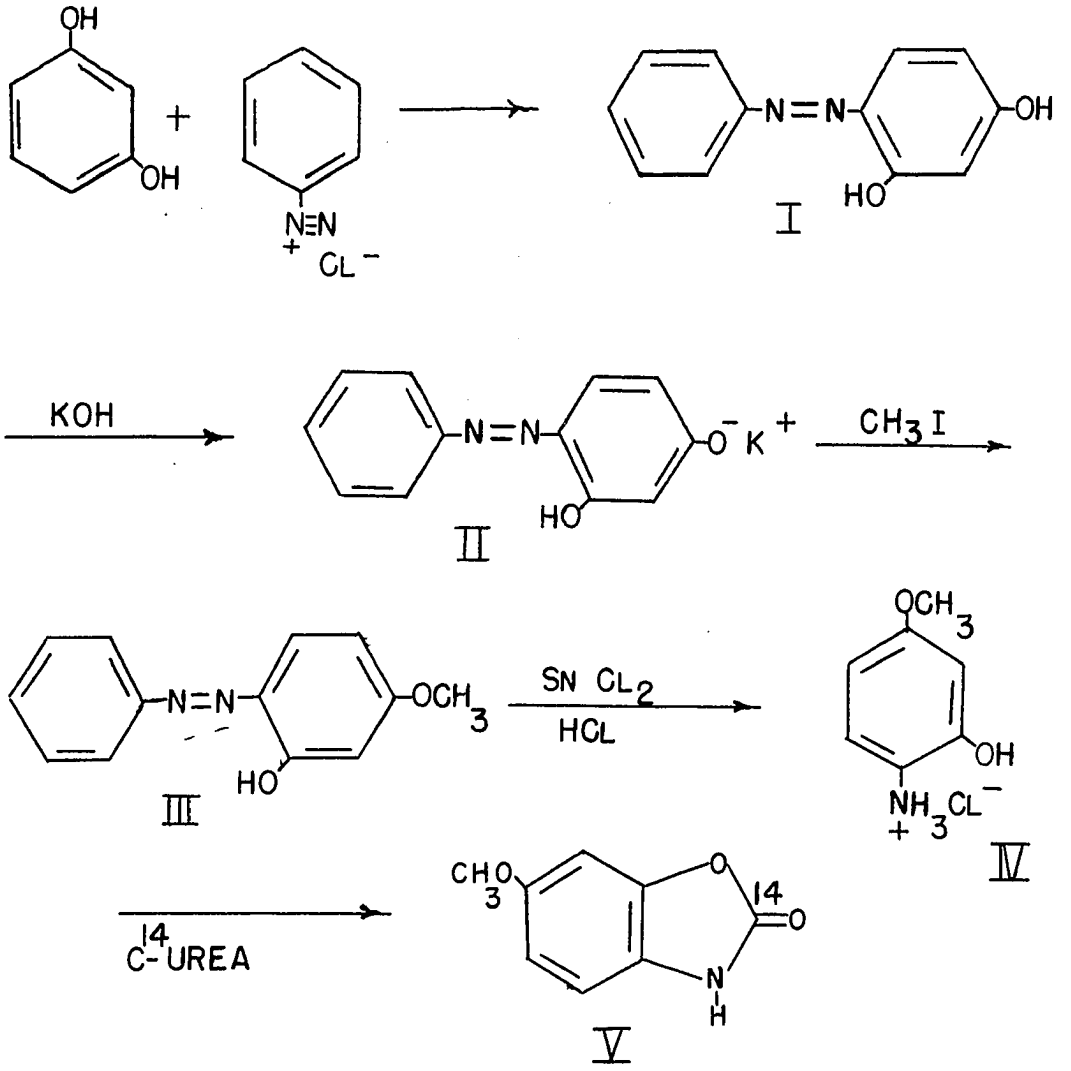
$$(X+Y)\,SA_2 = SA_1\,(Y)$$

$$X = \left(\frac{SA_1}{SA_2} - 1\right) Y$$

It can be seen that two specific activities (SA_1 and SA_2) and the weight of labeled compound added (Y) must be known to apply the technique. More rigorous treatment of the theory of isotope dilution and other applications of the technique are adequately presented by Gest *et al.* (1947), Overman and Clark (1960), and Kamen (1948).

Synthesis and Specific Activity Determination of 2-C^{14}-6-methoxy-2(3)-benzoxazolinone

The synthesis of 2-C^{14}-6-methoxy-2(3)-benzoxazolinone was carried out as a modification of those presented by Hietala and Wahlroos (1956) and Smissman et al. (1957b). The synthetic scheme is as follows:



p-benzene-azo-resorcine (I) was prepared by the diazotization reaction of benzenediazonium chloride with resorcinol according to Will and Pukall (1887). The reaction was found to have nearly quantitative yield. Recrystallization from absolute ethanol by the addition of water yields two different colored crystals each melting at different temperatures. A deep red crystal melting at 171°C was obtained by slow addition of water to an absolute ethanol solution of the compound. A yellow-orange crystal melting at 161°C was obtained when water was added rapidly to the absolute ethanol solution. It was found later that the 171°C melting compound formed its potassium salt (II) with a higher yield (18 per cent of theoretical) than did the 161°C melting compound (10 per cent of theoretical). Preparation of the potassium salt of p-benzene-azo-resoricine (II) according to the technique described by Will and Pukall (1887) proved to extremely unfruitful. A more feasible preparation of the salt was developed; stoichiometric amounts of potassim hydroxide pellets and p-benzene-azo-resorcine were placed in an erlenmeyer flask. Absolute ethanol was added until a slurry developed. The slurry was shaken for ten minutes and washed on a buchner funnel with absolute ethanol (yield 18 per cent). The methoxyl derivative (III) was then prepared using methyl iodide and potassium iodide according to Bechold (1889)

with a 15-20 percent yield. The methoxyl derivative melting point was 114°C. Reduction of the methoxy derivative (III) was carried out according to Henrich and Birkner (1913) with a thirty percent yield of 2-hydroxy-4-methoxyaniline (IV) melting at 196-200°C (decomposition). The amine hydrochloride (IV) was then reacted with C^{14}-urea in a fusion reaction described by Smissman et al. (1957b) yielding 2-C^{14}-6-methoxy-2(3)-Benzoxazolinone (V), melting point 151-152°C, UV spectra in 95% ethanol: Max. 229-230mμ and 285-286: Min. 255mμ. The infrared spectra of reaction scheme intermediates I, III, and the end product (V) are presented in Figure 1. The infrared spectrum of 2-amino-5-methoxyphenol was not prepared due its labile nature.

The fusion reaction with C^{14}urea was carried out twice. Once with 0.5 millicurie (synthesis A) and once with 1 millicurie (synthesis B) of C^{14}urea. Both C^{14}-urea samples were obtained from New England Nuclear Corporation, of Boston, Massachusetts. In each case, the 6MBOA product of the fusion reaction was recrystallized from water until constant specific activity was attained. Specific activity determinations were carried out utilizing a Packard model 314X Tri Carb Liquid Scintillation Spectrometer System. The scintillation solution used was "PPO-POPOP" in toluene (4 grams 2,5-diphenyloxazole, 100mg 1,4-di-(2-phenyloxazolyl)-benzene diluted to one liter with toluene). Deter-

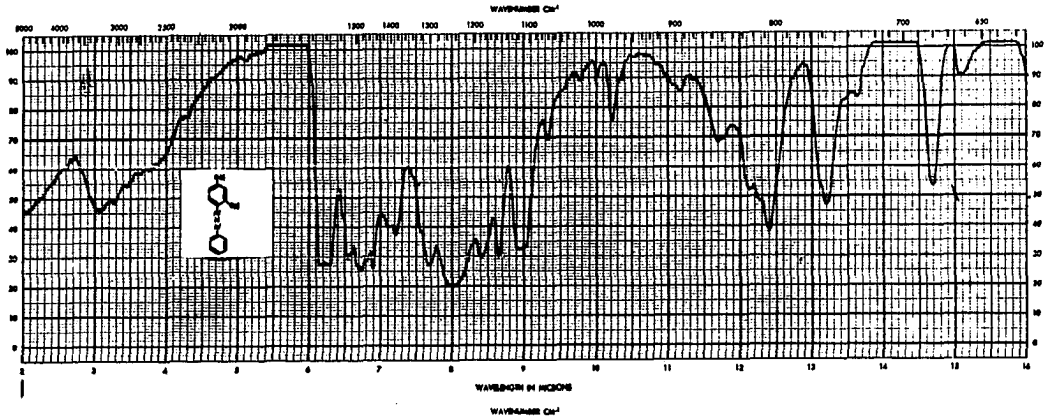
Figure 1. Infrared spectra of synthetic compounds and plant isolated 6MBOA (read from top to bottom)

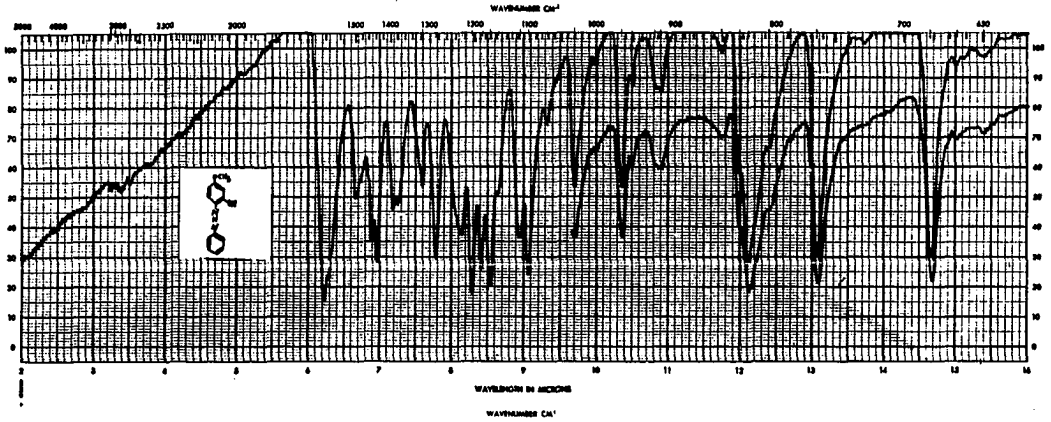
First - p-benzene-azo-resorcine (I)

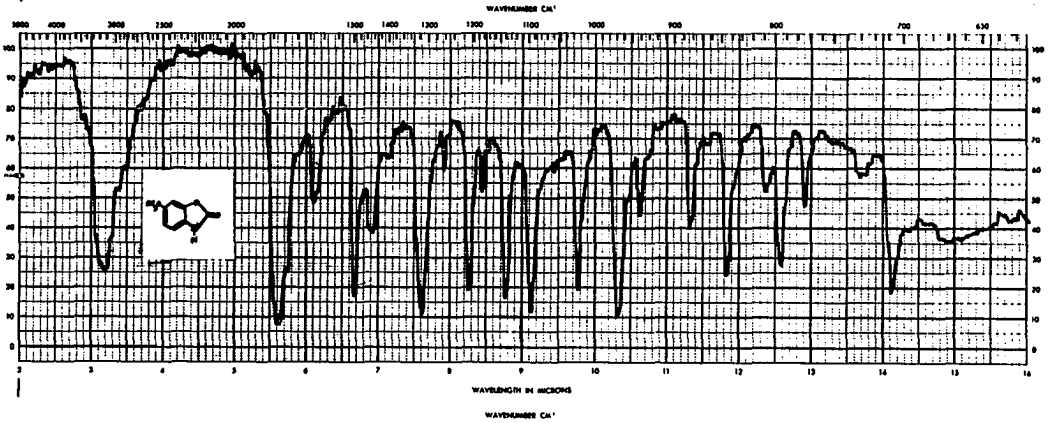
Second - p-benzene-p'-methoxy-azo-resorcine (III)

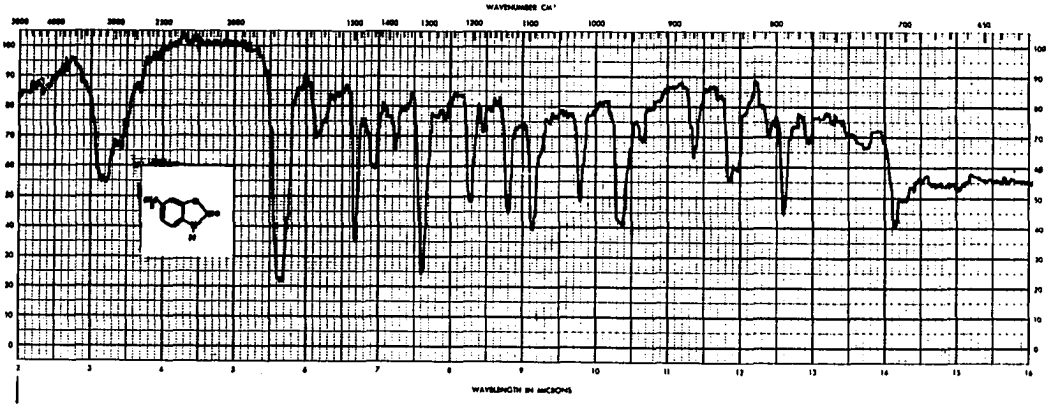
Third - 6-methoxy-2(3)-benzoxazolinone (from laboratory synthesis) (IV)

Fourth - 6-methoxy-2(3)-benzoxazolinone (isolated from plant tissue by thin layer chromatography)









mination of specific activity for a sample under analysis was carried out as follows. The C^{14}compound was weighed on a Mettler microbalance and then transfered to a ten ml. volumetric flask and filled to the mark with 95 per cent ethanol. Fifty microliters of C^{14}-6MBOA ethanol solution representing a known weight of labeled compound were then transfered to a liquid scintillation tube with a fifty microliter syringe. Ten ml. of scintillation solution were added to the scintillation tube containing the C^{14} sample. The tube was then placed into the liquid scintillation counter and counted for an interval of time sufficient to reduce the relative standard deviation of the counts/unit time to one per cent. The counts/unit time were corrected for background radiation and converted to disintegrations/unit time. Counter efficiency was determined by adding a C^{14}-toluene internal standard of known disintegrations/unit time/volume to the original sample. The sample internal standard was counted. The counts/unit time observed for the sample alone were subtracted from the sample internal standard counts/unit time. The remainder was divided by the disintegrations/unit time of the internal standard. The quotient represented the efficiency of the counter. The specific activity values of C^{14}-6MBOA for synthesis A and synthesis B, as ascertained

by repeated recrystallization and specific activity determinations, were 41.520 ∓ 0.530 μc/m $mole_{to0.05}$ and 76.336 ∓ 0.190 μc/m $mole_{to0.05}$ respectively.

Following the determination of specific activity, the compound was chromatographed on silica gel GF254 by thin layer chromatography utilizing three solvent systems; (1) chloroform: methanol(95:5) (2) ethylacetate: chloroform: cyclohexane(4:4:2) (3) ethanol. Each of the three chromatograms were viewed under short wave ultraviolet light and the C^{14}-6MBOA and co-chromatographed plant isolated 6MBOA were detected as single spots having identical Rf values. An autoradiograph of each chromatogram was prepared using Kodak X-ray tinted saftey base film. After seventeen days of exposure, the films were developed. No radiochemical was detected for synthesis A. For synthesis B, one spot corresponding to 6MBOA was detected on the chromatogram developed in EtOH and a trace of radiochemical impurity was detected for the chloroform: methanol(95:5) and ethylacetate: chloroform: cyclohexane(4:4:2) chromatograms. The impurity had a larger Rf value than that of 6MBOA in each case. The spots corresponding to impurity and C^{14}-6MBOA were eluted from the chromatograms with ethanol. The respective eluates were evaporated to dryness in vacuo and then taken up in 0.1 ml. ethanol. Fifty microliters of each solution was then counted. Results

are shown in Table 1 and indicate the trace of radiochemical impurity for synthesis B to be negligible.

Table 1. Demonstration of a negligible radiochemical impurity in synthesis B end product

Solvent System	Impurity cpm/50μl	C^{14}-6MBOA cpm/50μl
ethylacetate:chloroform: cyclohexane(4:4:2)	52	103,884
chloroform:methanol(95:5)	36	96,595

Quantitative Determination of 6-Methoxybenzoxazolinone

Quantitative analysis by the isotope dilution technique, or for that matter, any quantitative analysis of a system requires that extraction be complete. However, for the isotope dilution it is not required that recovery of the compound from the system be complete. It is assumed as extraction approach completion, SA_1 is diminished to SA_2 for the system.

Demonstration of the length of time required for complete extraction of 6MBOA from corn tissues was carried out as follows. Five hundred grams of dried ground whorl tissue of OH45 was placed on a table top. The mass was mixed thor-

oughly and then spread evenly into the form of a circle. Four 100 gram samples were taken, one from each quadrant. The remaining 100 gram portion was taken and utilized for a second two hour extraction test. Each of the 100 gram samples were placed into a three liter round bottom flask, two liters of water and boiling chips added, and a 95 per cent ethanol solution of C^{14}-6MBOA of known concentration pipetted into the mixture. Each of the flasks was fitted with a water cooled condenser, heated to boiling and allowed to reflux for 2, 4, 6, and 8 hours respectively after which the flasks were cooled to room temperature. 6MBOA was then isolated from each sample and specific activity SA_2 determined. Results of the analysis are shown in Table 2. It is to be noted that extraction is complete prior to the two hours of extraction time since SA_2 values for all extractions times are equivalent. Variation seen from one sample to the other is taken as an expression of sampling and handling error.

Table 2. Demonstration of complete extraction time of OH45 inbred tissue

Sample	Extraction time in hours	SA_2 μc/m mole	mg. 6MBOA/100 g. tissue
1	2	0.525	128.22
2	2	0.586	107.05
3	4	0.668	100.59
4	6	0.587	114.59
5	8	0.591	113.81

The specific activity SA_2 was determined as follows. A series of laboratory synthesized 6MBOA 95 per cent ethanol solutions was prepared. The absorbance of the samples at 230 mμ as compared with a reference 95 per cent ethanol solution utilizing a Beckman Model D U Spectrophotometer were plotted against the concentration of 6MBOA. The solutions of 6MBOA conformed to Beer's Law for an ideal solution over the range of concentrations utilized (0 to 20 x 10^{-3} mg./ml.). For an unknown sample, absorbancy was measured under the same conditions and was compared with the standard curve and its concentration read graphically. This technique was employed throughout the analysis.

Following isolation and purification of 6MBOA from any corn inbred by thin layer chromatography, the sample was taken up in 95 per cent ethanol. Fifty microliters of the solution were pipetted into a 10 ml. volumetric flask and 100 microliters into a liquid scintillation tube for counting. The 10 ml. volumetric was then filled to the mark with 95 per cent ethanol, its absorbancy measured at 230 mμ, and the concentration of the solution determined graphically from the standard curve. Thereby the weight of 6MBOA/50 microliters for the original solution was determined. Knowing the concentration of the original solution, it was then possible to calculate the weight of 6MBOA placed in the

liquid scintillation counter. Combining weight and disintegration rate values, an expression of disintegrations/minute/weight 6MBOA (SA_2) was obtained for the given sample.

A replicated experiment was run to determine the precision of the pipetting, Ultraviolet absorption, liquid scintillation counting procedure, utilized in determination of SA_2. Two samples of 6MBOA were used, one isolated from inbred OH45 and the other from the inbred R101. Results are tabulated in Table 3. It can be seen that relative deviation is less than 1 per cent, indicating a high degree of precision for the technique.

Table 3. Results of a replicated experiment designed to demonstrate precision of the technique used to determine the specific activity SA_2

Replicate	Specific Activity SA_2 in $\mu C/m$ mole
OH45-1	1.000
OH45-2	1.016
OH45-3	1.002
OH45-4	1.016
OH45-5	0.987
R101-1	1.730
R101-2	1.724
R101-3	1.751

Maize Inbreds and Isolation of 6-Methoxybenzoxazolinone from Maize Inbred Tissues

The eleven corn inbreds utilized in this study were chosen because as a series they represent the entire spectrum of resistance to the first brood European corn borer larvae from the highly resistant to the highly susceptible plant as determined by field ratings. The techniques utilized for the field evaluation of host plant resistance to the European corn borer (Guthrie 1960) and Dicke 1954) are based on a nine class rating scale: Class 1, resistant; Class 5, intermediate; Class 9, susceptible. Classification into a resistant, intermediate or susceptible class is dependent upon the size and shape of leaf injuries. Further differentiation within each class is determined by the number and extent of injuries. Resistance ratings for each inbred used in this analysis are expressed as grand mean ratings based on yearly mean ratings compiled by Iowa corn geneticists. These data are tabulated in Table 4.

Seeds of each inbred were obtained from corn geneticists at Iowa State University, Corn Improvement Research. Two plantings were carried out in this study, one in 1962 and the second in 1963. The inbred lines were planted in randomized blocks with one replication in 1963. In both years the seeds were planted on May 29 and samples taken

Table 4. Resistance ratings of inbreds to European corn borer attack, determined by field observation and compiled over a number of years; their respective grand means and standard deviations.

Inbred	Year	Mean Rating	Inbred	Year	Mean Rating	Inbred	Year	Mean Rating
C131A	1953[a]	1.5	Oh43	1953[a]	4.0	B14	1953[a]	7.0
	1954[a]	1.8		1954[a]	2.4		1954[a]	7.9
	1955[b]	2.0		1955[b]	6.0		1955[b]	7.0
	1956[c]	2.5		1956[c]	4.8		1956[c]	8.3
	1959[d]	2.2		1958[e]	5.7		1956[i]	7.4
	1961[f]	1.3		1959[d]	4.2		1958[e]	7.0
	1962[g]	2.3		1961[f]	5.0		1959[d]	7.4
	1963[h]	2.0		1962[g]	6.0		1961[f]	8.0
				1963[h]	4.0		1962[g]	8.3
Grand Mean		1.95					1963[h]	6.7
Standard Deviation		0.41	Grand Mean		4.67	Grand Mean		7.50
			Standard Deviation		1.15	Standard Deviation		0.59

[a]Source: (Penny 1955).
[b]Source: (Dicke 1955).
[c]Source: (Dicke 1957).
[d]Source: (Dicke 1959).
[e]Source: (Dicke 1958).
[f]Source: (Scott 1961).
[g]Source: (Scott 1962).
[h]Source: (Scott 1963).
[i]Source: (Penny and Dicke 1956).

Table 4. (Continued)

Inbred	Year	Mean Rating	Inbred	Year	Mean Rating	Inbred	Year	Mean Rating
B49	1959[d]	2.0	Oh45	1953[a]	3.0	B37	1955[b]	7.0
	1961[f]	1.7		1954[a]	3.4		1957[c]	8.3
	1962[g]	1.7		1955[b]	3.0		1958[e]	7.3
	1963[h]	3.0		1959[d]	4.0		1959[d]	6.7
							1961[f]	8.0
Grand Mean		2.10	Grand Mean		3.35		1962[g]	8.0
Standard Deviation		0.62	Standard Deviation		0.47		1963[h]	6.0
						Grand Mean		7.32
						Standard Deviation		0.83
B52	1958[e]	5.3	HY	1955[b]	4.0			
	1961[f]	5.0		1957[c]	5.3			
	1962[g]	6.7		1958[e]	5.3	WF9	1953[a]	8.0
							1954[a]	8.3
Grand Mean		5.66	Grand Mean		4.86		1959[d]	7.8
Standard Deviation		0.91	Standard Deviation		0.75		1961[f]	8.3
							1962[g]	8.3
							1963[h]	7.0
R101	1958[e]	5.4						
						Grand Mean		7.95
Grand Mean		___				Standard Deviation		0.51
Standard Deviation		___						

Table 4. (Continued)

Inbred	Year	Mean Rating	Inbred	Year	Mean Rating	Inbred	Year	Mean Rating
W22	1953[a]	4.3						
	1954[a]	5.5						
	1955[b]	5.0						
	1957[c]	6.0						
	1958[e]	7.3						
	1959[d]	3.0						
Grand Mean		5.18						
Standard Deviation		1.47						

for analysis thirty-nine days later. For each inbred the whorl portion of fifty plants were cut and then frozen in separate freezer containers. The whorl portion of the plant was chosen for 6MBOA analysis on the basis of reports by Dicke (1954) that young first brood larvae feed predominately on those tissues.

Preceeding isotope dilution analysis, tissues were thawed and dried in a vacumn oven at 38°-40°C. The dried samples were then ground to a fine powder in a Wiley mill.

The tissue to be analyzed for 6MBOA was weighed and placed in a three liter round bottom flask, two liters of water and boiling chips added, and a volume of 95 per cent ethanol solution of C^{14}-6MBOA of known concentration pipetted into the mixture. The mixture was refluxed for four hours and then cooled. The aqueous extract was filtered from the tissues on a Buchner funnel. The extract was then acidified to pH 2 with concentrated HCl and then extracted in a separatory funnel with three 300 ml. fractions of diethyl ether. The ether extract fractions were combined and evaporated to near-dryness in vacuo. The residue was taken up in 75 ml. ethylacetate:benzene (1:1), grade I Woelm alumina was added to the solution and mixed. The solution was then decanted and the alumina washed with 25 ml. ethylacetate:benzene (1:1). The two fractions were combined, centrifuged to remove particles of alumina, evaporated

in vacuo to a 2-3 ml. volume and placed in a teflon screw cap vial. This crude extract was then spotted in seventeen spots of approximately twenty microliters each across an edge of a 200 by 200 mm. thin layer of silica gel_{gf254} supported by a glass plate. The plate was then developed in an ascending solvent system of ethylacetate:chloroform: cyclohexane (4:4:2). After development was complete, the plate was observed under short wave ultraviolet light and 6MBOA detected by its Rf value corresponding to pure co-chromatographed 6MBOA. The area containing 6MBOA was then scraped off the plate with a stainless steel spatula and transfered to a 12 cm. pyrex centrifuge tube. The 6MBOA was eluted from the silica gel by flooding the silica gel with 5 ml. ethylacetate:benzene (1:1) and shaking. The mixture was then centrifuged and the resulting solution decanted into a 10 ml. evacuation bulb and evaporated to dryness using a rotatory evaporator. The residue was then taken up in fifty microliters of ethylacetate:benzene (1:1). The solution then was spotted and chromatographed on silica gel_{gf254} with chloroform:methanol (95:5) and the 6MBOA recovered as described above. The sample was then taken up in fifty microliters of 95 per cent ethanol, spotted once again on silica gel_{gf254}, and chromatographed with a cyclohexane:isobutanol (85:15) system. The compound was detected, that area of silica gel_{gf254} scraped from the

plate and eluted with 95 per cent ethanol, the solution centrifuged and evaporated to near-dryness in a rotatory evaporator. The remainder of ethanol was then allowed to evaporate slowly in a gentle stream of air, thereby yielding white needle-like crystals which were dried in a vacumn desiccator. The infrared spectrum prepared from the plant isolated crystals was found to be identical with that of laboratory synthesized 6MBOA (see Figure 1) and a melting point of 151°-152°C verified purity of the plant isolated compound as well as completing its identification as 6MBOA. Following the isolation of 6MBOA from plant tissues as described above, the specific activity (SA_2) was determined and the 6MBOA content of each inbred tissue calculated.

Bioassay of 6-Methoxybenzoxazolinone

Pure laboratory synthesized 6MBOA was bioassayed by incorporating the compound into an artificial European corn borer diet which was patterned after a European corn borer diet developed by Guthrie et al. (1965). The diet differed from the Guthrie et al. (1965) European corn borer diet in that brewers yeast was omitted and in certain cases modifications in vitamin constituents were made. However, in all other aspects, technique of preparation constituents, and proportions of constituents were the same. In all bioassay

tests of 6MBOA, the purified laboratory-synthesized compound was weighed and then dissolved in an appropriate volume of distilled water. The aqueous solution of 6MBOA was then utilized in preparation of the diet where an equivalent volume of water is required in normal diets. Thus control group diet and treated diet differed only in that distilled water alone was used as opposed to treated where an aqueous solution of 6MBOA was used. In all 6MBOA treatment groups, the aqueous solution was prepared such that a concentration of 0.5 mg. 6MBOA/gram of diet resulted. Assuming one gram of diet to be equivalent to one gram of fresh corn tissue, the concentration of 0.5 mg./g. diet is twice the concentration of that found in any of the inbreds analyzed for 6MBOA yield.

Eggs used in the various tests were produced by moths of a continuous rearing program at the U.S.D.A. European Corn Borer Research Laboratory, Ankeny, Iowa. Egg masses were incubated in screw-cap jars at 80°F. and 75 per cent relative humidity. After eggs had hatched, larvae were transferred individually into separate shell vials containing the test diet. A small camel's hair brush moistend with distilled water was used in the transfer. Vials were then plugged with cotton and incubated at 80°F. and 70 per cent relative humidity. Lights within the incubator remained on for 24 hours a day throughout the entire rearing

period so as to obviate the possibility of diapause. The larvae were observed daily and the rate of pupation and larval mortality recorded. After pupation had occurred individual pupae were weighed on a Roller-Smith balance and placed in snap-cap jars. All jars were then observed to determine sex and normalcy of moths. Moths were considered abnormal if morphological abnormalities were apparent. Thus, the criterion utilized for determining the effect of 6MBOA on the borer were larval mortality, pupation rate, weight of male and female pupae respectively, and condition of adult moths.

RESULTS

Correlation of 6-Methoxybenzoxazolinone with Inbred Resistance Rating

Results of the analyses of samples collected in 1962 and 1963 for the eleven inbreds are presented in Table 5. The quantity of 6MBOA found in the inbreds varied slightly from one season to the next but, relative quantities within the series remained constant. Highly resistant inbreds were found to contain approximately ten times more 6MBOA than the highly susceptible inbreds.

Table 5. The mean concentrations in milligrams of 6MBOA per 100 grams dry tissue found in eleven maize inbreds over the years 1962 and 1963

Inbred	Resistance rating	6MBOA/100g. 1962	6MBOA/100g. 1963	Average 6MBOA /100g. 62-63
		mg.	mg.	mg.
C131A	1.95	159.34	213.54	186.44
B49	2.10	202.67	246.67	224.67
OH45	3.35	84.12	102.72	93.42
OH43	4.67	48.24	63.42	55.83
HY	4.86	56.12	53.85	54.99
W22	5.18	91.01	135.98	113.50
R101	5.40		60.30	93.42
B52	5.66	55.60	68.15	61.88
B37	7.32	20.34	19.66	20.00
B14	7.50	20.43	32.29	31.26
WF9	7.95	24.81	16.63	20.72

The semilogarithmic plot of average 6MBOA concentrations for each inbred versus resistance rating of each inbred is shown in Figure 2. Standard deviation of resistance rating for each inbred and the observed variation of 6MBOA concentration for each inbred are represented. The plot indicates that 6MBOA concentration is correlated with inbred resistance according to the general equation

$$\text{Log}_{10}\ (\text{6MBOA conc.}) = -K(\text{resistance rating}) + B$$

and that larger quantities of 6MBOA are produced by inbreds which are more resistant to first brood European corn borer infestation. The line drawn for Figure 2 was constructed by application of the method of least squares.

In the course of analyzing the 1963 planting, tissue samples were thawed and then dried as needed. Analysis of the first replicate was carried out over a two month period after storage at -2°C for three months. The analysis of the second replicate was begun after tissues had been stored at -2°C for five months. There was no evidence of a change in 6MBOA yield from respective inbreds due to storage as may be seen in Table 6 which presents values obtained for the two replicates of 1963.

Figure 2. Relationship between 6MBOA yield and the resistance rating of eleven maize inbreds. Abscissa is resistance rating, 1 indicating a highly resistant inbred, 5 intermediate, and 9 a highly susceptible inbred. The ordinate is concentration, 6MBOA per hundred grams dry tissue, logarithmic scale.

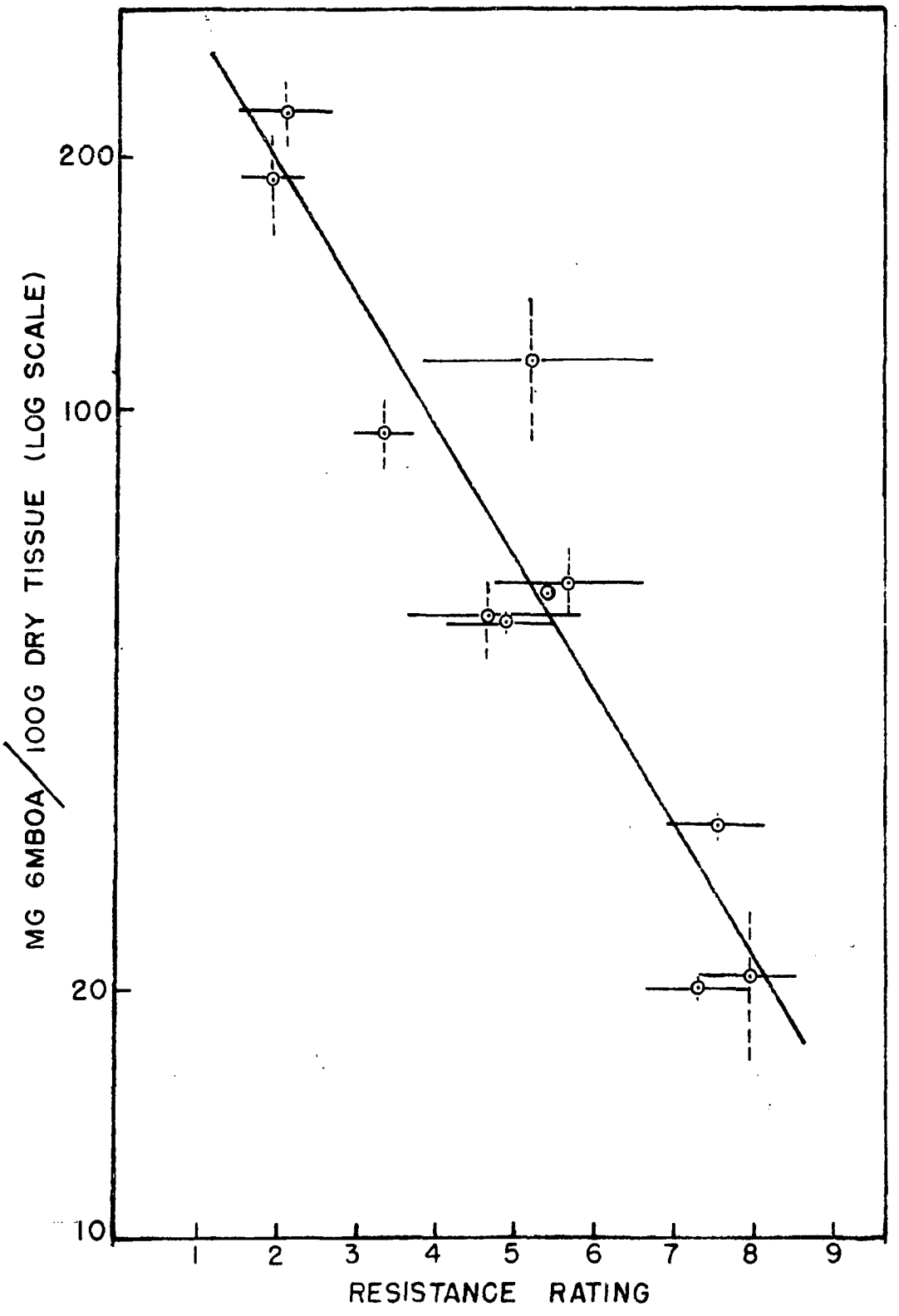

200
100
20
10
MG 6MBOA / 100G DRY TISSUE (LOG SCALE)
1
2
3
4
5
6
7
8
9
RESISTANCE RATING

Table 6. The concentration in milligrams of 6MBOA per 100 grams dry tissue found in two replicates which were analyzed in 1963. Tissues of replicate 1 were stored at -2°C for three months and tissues of replicate 2 were stored at -2°C for five months

Inbred	Rep. 1 6MBOA/100g.	Rep. 2 6MBOA/100g.
	mg.	mg.
C131A	202.58	224.50
B49	260.91	232.43
OH45	96.19	99.20
OH43	63.15	63.70
HY	47.48	60.23
W22	128.11	143.86
R101	54.92	65.70
B52	72.28	64.03
B37	18.71	20.62
B14	33.38	31.21
WF9	17.62	15.75

Earlier it was pointed out that controversy existed as to whether 6MBOA was in fact an *in vivo* constituent of uninjured maize tissue. (Smissman *et al.* 1963) and (Virtanen and Wahlroos 1963). Wahlroos and Virtanen (1959) have adequately demonstrated the degradation of the glucoside of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one to 6MBOA as fact. In addition, sufficient evidence has been presented by Virtanen and Wahlroos (1963), Brendenberg *et al.* (1962) and Reimann and Byerrum (1964) to

discount the presence of 6MBOA in uninjured maize tissues.

Preliminary analyses utilizing OH45 tissues indicated that acidification of the aqueous extract prior to ether extraction has no effect on yield of 6MBOA since addition of sodium bicarbonate as means of increasing solution polarity yielded the same 6MBOA concentration values as compared to acidified extracts. Thus, no 6MBOA is produced as the result of hydrolysis of the glucoside during the acidic-ether extraction and since there was no evidence of a change in 6MBOA yield from respective tissues due to storage, one may postulate that the hydrolytic enzyme (glucosidase) is stable at freezing temperatures for periods as long as seven months and that hydrolysis of the glucoside most likely occurs during or after tissues have thawed. Degradation of demethoxy-aglucone to BOA has been determined (Brendenberg 1962) to be a first order reaction. In a buffered solution, pH 7, 90°C, the first order rate constant is 4.05×10^{-1} min.$^{-1}$. A mixture of corn tissue and water, having been refluxed for four hours results in a system of pH 7. Thus, assuming the corn extraction system to be buffered at pH 7, the half life of the aglucone in the system may be estimated to be 1.71 min. It then follows that any aglucone present in the system at the start of the extraction procedure would be degraded to 6MBOA prior to the completion of the four hour extrac-

tion period and that all 6MBOA detected in the isotope dilution analysis would be the end product of glucosidase activity and the subsequent degradation of the aglucone. Analogously, the concentration of 6MBOA found in dried tissues is a measure of *in vivo* glucoside concentration.

Inhibition of Pupation Rate by 6-Methoxybenzoxazolinone and the Effect of Vitamins on 6-Methoxybenzoxazolinone Action

The compound 6MBOA was tested at a concentration of 0.5 mg./g. diet in a preliminary bioassay utilizing the European corn borer diet, developed by Guthrie *et al.* (1965). The pilot experiment indicated that 6MBOA had no significant inhibitory effect on borer development in this diet.

The results of Beck (1960) were reviewed. The "resistance diet" utilized by Beck did not include a vitamin supplement[1] as is used in the European corn borer diet of Guthrie *et al.* (1965). Beck noted that the vitamin supplement was deleted from the "resistance diet" on the basis of the assumption that brewers yeast was nutritionally satisfactory as a vitamin source. An I_{50} (concen-

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[1]Vitamin Diet Fortification Mixture purchasable from Nutritional Biochemicals Corporation.

tration of compound causing fifty per cent inhibition of borer growth based on larval weights) value of 0.429 mg. 6MBOA/g. diet was indicated by Beck (1960). It therefore was suspected that the inhibitory effect of 6MBOA was grossly attenuated by a certain vitamin or vitamins contained in the vitamin supplement.

Preceeding further bioassay, the European corn borer diet of Guthrie et al. 1965 was altered as follows. Brewers yeast was removed and five vitamins found in the vitamin supplement were selected to be included in the altered diet. Vitamins selected were those which, if found in fresh maize tissue, would most likely be absent in the dried and ground WF9 maize inbred tissue utilized as a "corn leaf factor" source in the borer diet due to their labile nature. The five vitamins selected were calcium pantothenate, thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and folic acid.

Utilizing the five vitamin diet, 6MBOA was bioassayed. Forty-five larvae were placed on the control (5 Vitamins) and treated (5 Vitamins + 6MBOA) diets respectively with one replication in each case. Results are presented in Table 7. Pupal weight data were analyzed statistically applying an unweighted mean analysis and a planned orthogonal comparison. The result of the analysis is presented in Table 8. These data show that no significant difference in pupal

Table 7. Mean pupal weights of European corn borer reared in bioassay tests (males and females respectively)

Treatment	Female weight	n[1] [a]	Male weight	n[1]
	mg.		mg.	
	Replicate 1			
5 Vit	93.57	17	68.28	21
5 Vit + 6MBOA	86.08	16	71.11	21
5 Vit + B12	83.10	20	67.94	16
5 Vit + B12 + 6MBOA	89.48	13	62.41	16
Vit Sup	114.90	30	80.53	15
Vit Sup + 6MBOA	114.35	20	83.54	25
	Replicate 2			
5 Vit	82.62	17	65.21	15
5 Vit + 6MBOA	84.64	15	65.15	13
5 Vit + B12	89.75	16	64.64	18
5 Vit + B12 + 6MBOA	86.87	13	65.63	17
Vit Sup	113.83	25	80.07	20
Vit Sup + 6MBOA	112.73	19	81.27	24

[a] n^1 - number of individuals.

weight was detected between the treatment and control diets. However, a rather striking retardation in rate of pupation was shown in the presence of 6MBOA as is shown in Figure 3. No significant larval mortality was observed between the treated control groups. Larvae on the diet containing the

Table 8. The analysis of variance and orthogonal comparison of bioassay tests based on pupal weights

Source	D.f.	Sum of Squares	Mean Square	$F_{0.01}$
Replications	1	21.81	21.81	n.s.[a]
Diets	5	2,356.23	471.25	8.31**
1 vs 2[b]	1	0.91		n.s
3 vs 4	1	0.14		n.s
5 vs 6	1	0.81		n.s
$\frac{1\ 2}{2} - \frac{5\ 6}{2}$	1	1,692.50		**
remainder	1	661.87		
Sex	1	3,654.12	3,654.12	64.42**
Treatment by sex interaction	5	217.27	43.45	n.s.
Error	418	23,708.96	56.72	

[a] n.s. = not significant
** = highly significant at 1%

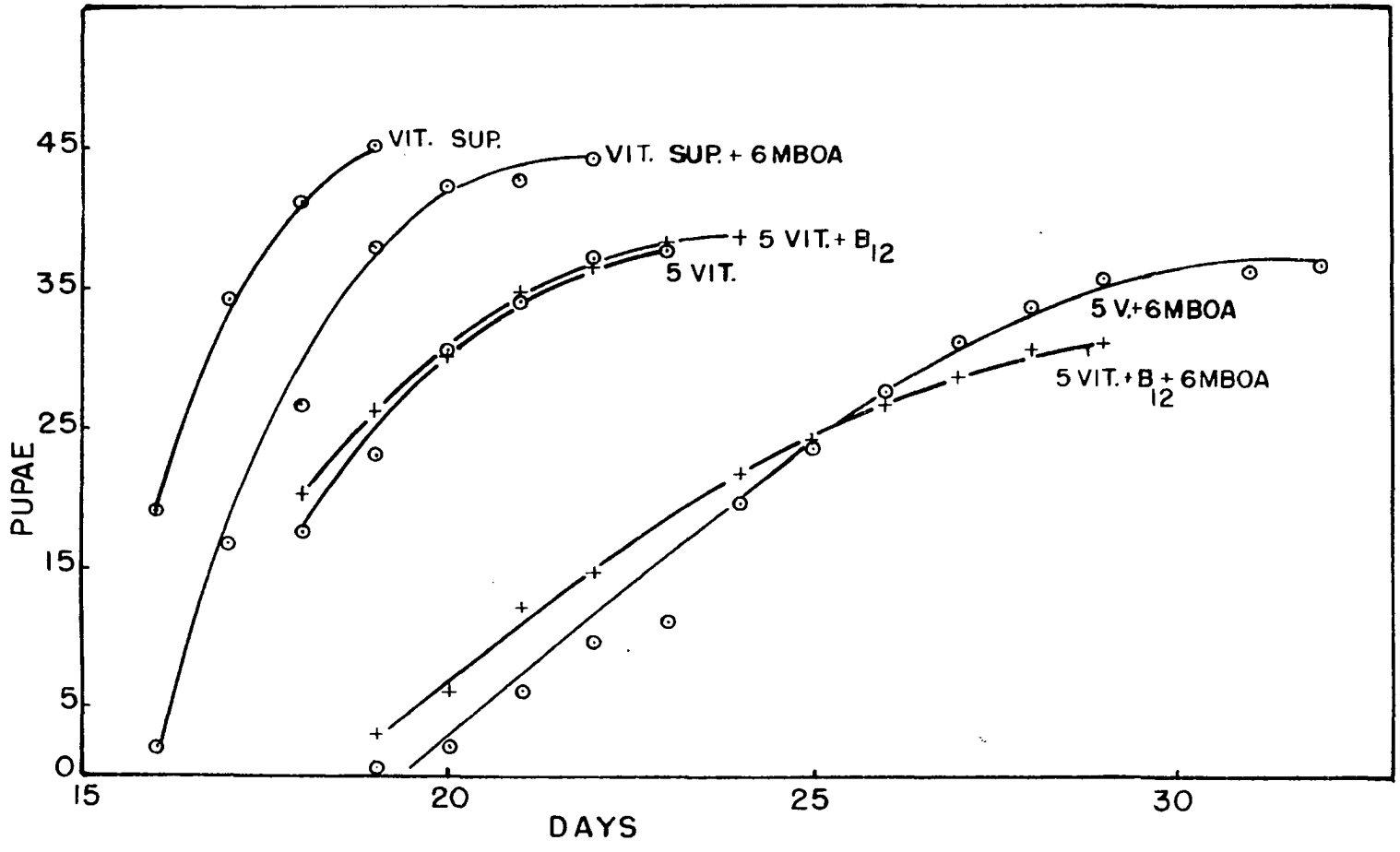
[b] Orthogonal comparisons:

1 vs 2 = 5 Vit vs 5 Vit + 6MBOA
3 vs 4 = 5 Vit + B_{12} vs 5 Vit + B_{12} + 6MBOA
5 vs 6 = 5 Vit sup. vs Vit sup + 6MBOA
$\frac{1\ 2}{2} - \frac{5\ 6}{2}$ = 5 Vit vs Vit supplement

five vitamins completed pupation within a period of six days while the larvae on the diet containing the five vitamins plus 6MBOA required fourteen days for completion of

Figure 3. Effect of 6MBOA upon pupation rate and demonstration of the action of a vitamin supplement on 6MBOA effect. A total of ninety larvae in two replicates were utilized for each test diet with forty-five larvae on each replicate. Pupae values are the cumulative average number of pupae over the two replicates; abscissa is number of days from the time larvae were placed on each diet.

Vit. Sup.:	Vitamin Supplement
5 V. or 5 Vit.:	5 Vitamins
B_{12}:	Vitamin B_{12}
6MBOA:	6-methoxybenzoxazolinone


VIT. SUP.
VIT. SUP. + 6MBOA
+ 5 VIT. + B12
5 VIT.
5 V.+6MBOA
5 VIT. + B12 + 6MBOA
PUPAE
DAYS
0
5
15
25
35
45
15
20
25
30

pupation. A similar bioassay test utilizing the five vitamins plus vitamin B_{12} was also run. Results of the test were equivalent to those obtained where only five vitamins were utilized as may be verified by reviewing that data shown in Table 7, Table 8, and Figure 3. In both of the preceeding bioassay tests, no significant morphological abnormality was noted for treated groups relative to controls. The compound 6MBOA was then bioassayed in a diet which utilized the vitamin supplement as a vitamin source. Once again, forty-five larvae were placed on the control (vitamin supplement) and treated (vitamin supplement + 6MBOA) diets respectively with one replication in each case. The composition of the vitamin supplement is presented in Table 9.

No significant difference in pupal weight was detected between pupae reared on the control diet and those grown on the treated diet. Borer pupal weight for the experiment was found to be significantly higher than that found when only five vitamins were utilized. These data are presented in Table 7 and Table 8. The data shown in Figure 3 indicates that the effect of 6MBOA upon the pupation rate observed in the five vitamin diet has been grossly attenuated. Pupation began three days earlier and was completed ten days sooner on the vitamin supplement plus 6MBOA diet than that observed for the five vitamins plus 6MBOA diet. Survival

Table 9. Composition of the vitamin supplement[a]

Constituent	mg./100g diet
Group 1	
Ascorbic acid	15.80
Choline chloride	26.25
Riboflavin	0.35
Pyridoxine	0.35
Folic acid	0.0315
B_{12}	0.0047
Calcium pantothenate	1.05
Thiamine	0.35
Group 2	
Inositol	1.75
Niacin	1.58
Biotin	0.007
alpha-tocopherol	1.75
Vitamin A (2×10^5 units/g.)	1.575
Vitamin D (4×10^5 units/g.)	0.875
Menadione	0.0875
para-aminobenzoic acid	1.75

[a] Nutritional Biochemicals Corporation's Vitamin Diet Fortification Mixture.

in the presence of the vitamin supplement both for treated and control was higher than that found where only five vitamins were supplied.

Vitamin constituents of the vitamin supplement can be divided into two separate groups. The division into Group 1 and Group 2 is presented in Table 9. The first group is

composed of those vitamins which when incorporated into a borer diet exhibit no attenuative action to 6MBOA effect. Group 2 is composed of those vitamins which when utilized in the borer diet in combination with Group 1 results in gross attenuation of 6MBOA effect. Identification of that vitamin or specific vitamin combination which attenuates the effect of 6MBOA was not attempted.

SUMMARY AND DISCUSSION

The primary objective of this research was to evaluate the role of 6MBOA in the resistance of maize inbreds to the European corn borer. From the results obtained in the course of research, the following conclusions were drawn.

The yield of 6MBOA by maize inbred tissues can serve as an indicator of the degree of resistance one may expect a given inbred to express in the field. The highest concentration of 6MBOA in dried tissues was found in the resistant inbred B_{49}. The inbred yielded 224.67 mg./100 g. dry tissue which is equivalent to approximately 0.22 mg./g. fresh tissue. The lowest concentration of 6MBOA in dried tissues, 20 mg./100 g. dry tissue, was found in the susceptible inbred B37. This concentration is equivalent to approximately 0.02 mg./g. fresh tissue. For the entire inbred series of eleven inbreds, the correlation of 6MBOA concentration with resistance rating can be expressed by the general equation

$$\text{Log}_{10}(\text{conc. 6MBOA}) = -K(\text{resistance rating}) + B.$$

Since 6MBOA is produced upon degradation of a glucoside precursor, the 6MBOA yield values from the various tissues is also a direct measure of glucoside precursor content of

uninjured tissues.

Resistance to the first brood European corn borer is characterized by the fact that larval survival is low on resistant inbreds. Bioassay tests have demonstrated that 6MBOA does not cause significant larval mortality, has no effect on pupal weight, and is biologically active only in that rate of pupation is inhibited when the compound is incorporated into a borer diet at a concentration of 0.5 mg./g. diet. Addition of a vitamin supplement to a diet possessing the same 6MBOA concentration results in a gross attenuation of this effect. Inhibition of pupation rate may also be interpreted as inhibition of larval development. Inhibition of larval development was the criterion utilized by Beck (1960) where he presented an I_{50} value of 0.429 mg./g. diet.

Quantitative analysis of the series of inbreds for 6MBOA has shown that the highest concentration of 6MBOA yielded by a resistant inbred is less than one-half that concentration applied in bioassay tests, assuming one gram of artificial diet to be equivalent to one gram of fresh corn tissue. Assuming further that the vitamins of Group 2 (those antagonistic to 6MBOA) are absent in maize tissues, the greatest inhibitory effect the compound would have on borers feeding on such tissues would be expected to be substantially less than that observed in bioassay

tests where twice the concentration of 6MBOA found in any tissue was utilized. In addition, if the vitamins of Groups 1 and 2 are present in corn tissue, the expected effect of 6MBOA on the European corn borer would be trivial. It therefore must be concluded that 6MBOA plays only a subordinate role in the resistance phenomenon. However, due to the observed correlation of 6MBOA (a compound formed from a glucoside precursor) with resistance ratings, it may well be that the precursors of 6MBOA are more active biologically than 6MBOA and since their activity has not been determined experimentally, they cannot be discounted as possible borer toxins or growth inhibitors.

The correlation between resistance and yield of 6MBOA or glucoside precursor content evidences a differential in alkaloid biosynthesis for the inbred series and similarily demonstrates the existence of a difference in biosynthetic pathway for the series. It follows that a similar differential production of various yet unisolated toxic or inhibitory compounds may exist in maize tissues and like the glucoside and aglucone precursors of 6MBOA may be labile in nature. Such labile unisolated compounds may possess highly active biological properties and play an important role in the resistance of maize to the European corn borer.

George (1957) has shown that the incorporation of resistant inbred tissues in a borer diet does not result in

inhibition of borer development as is observed when borers are placed on resistant plants in the field. It must be noted however, that in these bioassay tests, the maize tissues were frozen, thawed, dried, and ground prior to their incorporation in the diet. Such treatment of tissues drastically alters chemical constituents and it may be expected that labile biologically active components which possibly exist in undisrupted plant tissue would be degraded to inactive forms as the result of enzymic and chemical degradative processes. As a result one would not expect to observe inhibition of borer development in such bioassay procedures.

Efforts by other workers to extract biologically active components from maize tissues have also been abortive. Here again, attempts were not made to denature plant enzymes prior to extraction procedures and many of the extraction techniques employed were severe. Need for the development of specialized techniques for the isolation of labile components which include a process of plant enzyme denaturation and less severe extraction procedures is evident. An approach to the problem which encompasses this point of view, may result in the isolation of plant chemicals responsible for resistance which heretofore have eluded researchers.

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